A STUDY OF RESAZURIN REDUCTION IN FRESHLY DRAWN MASTITIC-LIKE MILK

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INTRODUCTION

Mastitis is an inflammation of the mammary gland. It is generally believed to be caused by bacterial infection, by injury to the teat or udder tissue, or both of these factors. The physical symptoms consist of glandular abnormalities, including inflammation, swelling, and tenderness; and visible abnormalities of the milk, including clots, viscous serum, and even blood. These are dependable criteria in the detection of acute infections and advanced cases of chronic mastitis, but they do not provide accurate means of diagnosis either of the early stages of infections or of the specific infecting agent.

Detailed diagnosis requires the examination and analysis of milk from the individual quarters of the udder. The presence of incriminating bacteria in the fore-milk is the primary indication of infectious mastitis. This condition, accompanied by large numbers of leucocytes, high concentrations of chlorides, and increases in catalase and pH values, is an indication of an established infection.

The resazurin test was included in the ninth edition of Standard Methods for the Examination of Dairy Products as an official technique for the grading of raw milk samples. While this test was first introduced into this country by Ramsdell in 1935, one factor possibly more responsible than any other for delaying its official acceptance was its sensitivity to pathologically abnormal and pathological milks.

If the resazurin test, which is well adapted to farm use through Goldings' dry vial modification, can be established as a valuable field test for the detection of mastitis, those working on the control of this disease will have a rapid means of examination of the individual quarters.

The object of this study was to establish the degree of correlation existing between resazurin reduction in freshly drawn quarter samples of milk and tests which have already been accepted for the detection of mastitis. If such a correlation can be established, the resazurin test will then be suitable for the rapid detection of mastitis on the farm, as well as for the routine grading of raw milk at the plant.

REVIEW OF LITERATURE

A complete review of literature relating to mastitis infection and diagnosis was not attempted, due to the tremendous volumes that have been written on this subject. The following, therefore, is intended to familiarize the reader with the resazurin test and the chemical and bacteriological tests used in this study.

In the routine grading of raw milk supplies, the resazurin test is run on milk samples lifted at the weigh tank, and usually consists of a composite sample of mixed night and morning milk. Reduction of the dye depends upon the kind and number of bacteria present and the degree of abnormality. When milk is normal, with a low bacterial count, the reduction will be gradual from the original blue to white. When the bacterial count is high, that is in terms of millions, the reduction will be rapid, going from the original blue through pink to white. The same will be true of milk having a high bacterial count accompanied by large numbers of leucocytes. On the other hand, when the bacterial count is low and there is a high leucocyte count, there will according to Davis, Johns, Little and Ramsdell be a rapid reduction to pink followed by a lag phase, with the final reduction to white being brought about by the gradual increase in bacterial numbers.

Davis claims that when the resazurin test is run on milk that is less than four hours old, the resazurin reduction is a measure of leucocyte activity only.

According to Burkey, normal milk from a healthy udder is free from mastitic-causing organisms, contains relatively few leucocytes, contains less than 0.10 percent chlorides, and has a pH value of about 6.5. The presence of excessive numbers of leucocytes is an indication of inflammation and is the basis of the catalase test. An arbitrary number of 100,000 leucocytes per ml has been considered by some investigators as the upper limit for normal milk, while Little has designated 300,000, and Hucker and Burkey 500,000. Rosell found that a concentration of chlorides greater than 0.14 percent and a pH value of 6.8 or higher is indicative of mastitis.

It is clear from the above that the tests for abnormal milk or mastitis are a measure of degree, rather than a post-
itive statement of the presence or absence of infection.

**Experimental.**

**Presumptive screening.** To avoid the unnecessary work and loss of time through the routine examination of many samples of normal milk, a screening procedure was adopted using catalase and pH determinations in preliminary examination. Where quarter samples showed either a gas production of 10 percent or more by the catalase test, or a pH value of 6.65 or higher, the milk from that quarter was routinely examined using five confirmative tests.

**Confirmative testing.** The composition of mastitic milk changes very rapidly. Because of this, the fresh samples for the confirmative tests were run as soon as possible after screening, usually within 24 hours. The confirmative tests included: the leucocyte count, chloride, catalase and pH determinations, and the hemolytic count on blood agar. For supporting information, it was thought desirable to make standard plate counts on all samples examined.

**Sampling.** The samples for screening, and for confirmative testing, were collected from each quarter at the beginning of the evening milking. With one exception, the quarters showed no signs of physical abnormalities. Theudder, flank, and teats were washed with water containing a disinfectant and then wiped practically dry. The first three streams were discarded from each quarter, after which a sterile 3-ounce screw-capped sample bottle was filled directly from the teat and capped immediately. The samples were placed in a box containing ice and taken immediately to the laboratory. At no time did more than one and one-half hours lapse from the time that the first samples were drawn until testing was begun.

The resazurin test, using Golding's dry vial modification, the standard plate count, and the hemolytic counts was made according to the procedure outlined in Standard Methods. The chloride determinations were made by measuring the electrical conductivity of the milk, using McCulloch's electro- metric method. The procedure for the catalase test was the same as adopted by Halversen. A Beckman industrial potentiometer was used for determining the pH values.

The following criteria were used to present an evaluation of mastitic conditions as determined by the examination of samples of fore-milk from individual quarters: Mastitic or abnormal milk was considered to be milk containing 100 (lower limit of quantitative count used) or more hemolytic colonies per ml on blood agar, 500,000 or more leucocytes per ml, 16.6 percent or more catalase, 0.10 percent or more of chlorides, and milk with a pH value of 6.8 or higher.

**Experimental Results**

In all, 985 presumptive screening tests were run. This screening procedure showed that 269 quarters were producing milk of a mastitic-like nature. The resazurin triple-reading and confirmative tests were run on the milk from these quarters.

The results of the resazurin reduction at the end of one, two, and three hours incubation, compared with the means for the confirmative tests, are shown in table 1.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>No. of samples</th>
<th>Catalase percent</th>
<th>pH</th>
<th>Chlorides percent</th>
<th>Leucocytes (ml)</th>
<th>Hemolytic count</th>
<th>Standard plate count</th>
</tr>
</thead>
<tbody>
<tr>
<td>One hour</td>
<td>1</td>
<td>61</td>
<td>6.67</td>
<td>0.085</td>
<td>156</td>
<td>734</td>
<td>1,411</td>
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<tr>
<td></td>
<td>2</td>
<td>26</td>
<td>6.68</td>
<td>0.095</td>
<td>156</td>
<td>3,458</td>
<td>9,220</td>
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<tr>
<td></td>
<td>3</td>
<td>60</td>
<td>6.78</td>
<td>0.111</td>
<td>2,128</td>
<td>4,852</td>
<td>14,955</td>
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<tr>
<td></td>
<td>4</td>
<td>55</td>
<td>6.81</td>
<td>0.125</td>
<td>5,501</td>
<td>19,173</td>
<td>23,726</td>
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<tr>
<td></td>
<td>5</td>
<td>25</td>
<td>6.90</td>
<td>0.161</td>
<td>19,604</td>
<td>42,732</td>
<td>68,640</td>
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<tr>
<td>Two hour</td>
<td>1</td>
<td>29</td>
<td>6.69</td>
<td>0.084</td>
<td>156</td>
<td>510</td>
<td>1,288</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>62</td>
<td>6.65</td>
<td>0.091</td>
<td>156</td>
<td>3,458</td>
<td>9,220</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>42</td>
<td>6.71</td>
<td>0.092</td>
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<td>6,564</td>
<td>12,164</td>
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<tr>
<td></td>
<td>4</td>
<td>83</td>
<td>6.77</td>
<td>0.112</td>
<td>2,890</td>
<td>8,716</td>
<td>14,687</td>
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<tr>
<td></td>
<td>5</td>
<td>61</td>
<td>6.80</td>
<td>0.150</td>
<td>25,687</td>
<td>30,632</td>
<td>48,030</td>
</tr>
<tr>
<td>Three hour</td>
<td>1</td>
<td>3</td>
<td>6.63</td>
<td>0.085</td>
<td>26</td>
<td>100</td>
<td>2,300</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>56</td>
<td>6.67</td>
<td>0.085</td>
<td>123</td>
<td>616</td>
<td>1,546</td>
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<tr>
<td></td>
<td>3</td>
<td>44</td>
<td>6.67</td>
<td>0.086</td>
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<td>1,518</td>
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<tr>
<td></td>
<td>4</td>
<td>99</td>
<td>6.74</td>
<td>0.103</td>
<td>2,062</td>
<td>9,134</td>
<td>16,851</td>
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<tr>
<td></td>
<td>5</td>
<td>67</td>
<td>6.82</td>
<td>0.145</td>
<td>11,315</td>
<td>25,086</td>
<td>39,205</td>
</tr>
</tbody>
</table>

As the reduction of resazurin in milk was continued from one to three hours, there was a gradual lowering of the means for the confirmative tests when compared with the means of these at the previous hour. This is no doubt due to the inclusion in the higher resazurin readings at the second and third hour of samples that show a lesser degree of abnormality than those samples considered positive by the resazurin after one hour incubation. The number of samples with a resazurin reading of 1 after one hour incubation was 61, compared with 29 after two hours and 3 after three hours incubation. The number of samples with a resazurin reading of 5 increases with each additional hour incubated.

On the basis of a one hour incubation and a resazurin reading of 5, the resazurin test identifies the more severe cases of sub-clinical mastitis.

Twenty-five samples were completely reduced to 5 within one hour. From data not presented, two of these were completely reduced to white within ten minutes. In neither case was there a standard plate count exceeding 6,100 bacteria per ml, even though they both had exceedingly high leucocyte counts and were abnormal in appearance.

In table 2 will be found the range for the confirmative tests at each incubation period and for each reading in the resazurin reduction.

A comparison of the confirmative tests with resazurin readings at different incubation times can best be made from a study of table 3. From these results, it is apparent that the catalase...
TABLE 2

Comparation of Resazurin Readings with the range for the confirmative tests

<table>
<thead>
<tr>
<th>Period of incubation</th>
<th>Resazurin reading</th>
<th>Catalase percent</th>
<th>pH</th>
<th>Chlorides</th>
<th>Leucocytes (100)</th>
<th>Hemolytic count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of samples</td>
<td>rate of 6.45-6.72</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One-hour incubation</td>
<td>1 61</td>
<td>6.45-6.72</td>
<td>6.45-6.72</td>
<td>0-300</td>
<td>0-156</td>
<td>100-15,700</td>
</tr>
<tr>
<td></td>
<td>2 70</td>
<td>6.45-6.89</td>
<td>0-300</td>
<td>0-925</td>
<td>100-15,680</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 80</td>
<td>6.45-6.67</td>
<td>0-300</td>
<td>0-925</td>
<td>100-15,700</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 90</td>
<td>6.45-6.67</td>
<td>0-300</td>
<td>0-925</td>
<td>100-15,680</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 100</td>
<td>6.45-6.67</td>
<td>0-300</td>
<td>0-925</td>
<td>100-15,700</td>
<td></td>
</tr>
</tbody>
</table>

**Highest limit of quantitative determination
**Lower limit of quantitative count used.

From the data presented in table 4, it is evident that the correlation between resazurin reduction and the confirmative tests used is highly significant at the 1 percent level.

**Discussion**

Several investigators have emphasized the importance of leucocytes in detecting mastitis. From the data presented in this study, it is apparent that resazurin is very sensitive to freshly drawn milk containing large numbers of leucocytes. When a three-hour incubation time and a resazurin reading of 3 is used as a confirmative test, this test will detect 98.4 percent of those samples which had a leucocyte count of 500,000 or more per ml. It should be noted, however, that the basis of this criterion the mean leucocyte count was 712,000 per ml. While resazurin detected 28 more samples than considered positive on the basis equally sensitive when compared with the confirmative tests in differentiating between normal and abnormal milk. Little advantage was gained from the longer incubation time. If a three-hour incubation and a resazurin reading of 3 or mauve-pink be chosen as a criterion for differentiating between mastitic and normal milk, then 96.7 percent of the quarters were positive on the basis of the catalase test, 95.2 percent on the basis of pH, 92.7 percent on the basis of chlorides, 98.4 percent on the basis of leucocytes, and 90.8 percent on the basis of the hemolytic count. Using the same criterion, 210 quarters would have been graded positive by the resazurin test compared with 181 by catalase, 79 by pH, 128 by chloride, 182 by leucocytes, and 149 by the hemolytic count. Resazurin seems to be more sensitive to this abnormal milk than any of the confirmative tests used.

TABLE 4

Comparison of the correlations of the Resazurin Readings With Confirmative Tests

<table>
<thead>
<tr>
<th>Incubation in hours</th>
<th>Percent catalase</th>
<th>pH</th>
<th>Percent chloride</th>
<th>Leucocyte count</th>
<th>Hemolytic count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+0.450*</td>
<td>-0.794</td>
<td>+0.647</td>
<td>+0.571</td>
<td>+0.309</td>
</tr>
<tr>
<td>2</td>
<td>+0.465</td>
<td>+0.810</td>
<td>+0.630</td>
<td>+0.479</td>
<td>+0.382</td>
</tr>
<tr>
<td>3</td>
<td>+0.462</td>
<td>+0.798</td>
<td>+0.589</td>
<td>+0.469</td>
<td>+0.630</td>
</tr>
</tbody>
</table>

* * n equals 219, otherwise the n value was 269.
The lag phase reported by Davis, Johns, Ramsdell, and Little to occur during the reduction of this dye in milk containing large numbers of leucocytes was not observed in the 25 samples which were completely reduced to white in one hour. The fact that in no case was the standard plate count above 800,000 bacteria per ml eliminates any possibility of bacteria completing the reduction of the dye. The fact that two samples which were completely reduced in ten minutes had plate counts of 6,100 bacteria per ml or less substantiates this.

The observation made by Davis that when the resazurin test is run on milk samples within four hours of milking, it is a measure of leucocyte activity only, was confirmed in this study.

The close relationship found by Ramsdell to exist between resazurin reduction and catalase was evident in this study, since the resazurin test detected 96.7 percent of the samples that were positive on the basis of the catalase test. This correlation is important since Rosell claims that the catalase test will detect from 90 percent to 95 percent of the samples that, when using a three-hour incubation, or preferably a resazurin modification when supervised by a portable electrometric device, would be diagnosed as mastitic is a standard, 15 quarters having a hemolytic count greater than 0.12 percent chlorides as positive mastitis.

1. When the resazurin test is run on quarter samples of milk within four hours of milking, it is a measure of leucocyte activity only.
2. Resazurin dye in freshly drawn quarter samples of mastitic-like milk with the confirmative test used is highly significant.
3. Resazurin dye in freshly drawn quarter samples of mastitic-like milk may be reduced to colorless, and the lag phase as previously reported is not noted under such conditions.
4. The bacteria present in such mastitic-like milk are not sufficient to affect the reduction.
5. A resazurin reading of 3 on a one-hour incubation, or preferably a resazurin reading of 3 on three-hour incubation, can be used as a suitable criterion for detecting cases of subclinical mastitis.
6. The resazurin test, using the dry vial modification when supervised by a competent fieldman, can be used to advantage on the farm as a screening test for mastitis.

**Conclusions**

1. The correlation of resazurin reduction in freshly drawn quarter samples of mastitic-like milk with the confirmative test used is highly significant.
2. When the resazurin test is run on quarter samples of milk within four hours of milking, it is a measure of leucocyte activity only.
3. Resazurin dye in freshly drawn quarter samples of mastitic-like milk may be reduced to colorless, and the lag phase as previously reported is not noted under such conditions.
4. The bacteria present in such mastitic-like milk are not sufficient to affect the reduction.
5. A resazurin reading of 3 on a one-hour incubation, or preferably a resazurin reading of 3 on three-hour incubation, can be used as a suitable criterion for detecting cases of subclinical mastitis.
6. The resazurin test, using the dry vial modification when supervised by a competent fieldman, can be used to advantage on the farm as a screening test for mastitis.

**References**